

## Lipoarabinomannan and Lipid-Free Arabinomannan Antigens of *Mycobacterium paratuberculosis*†

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Lipoarabinomannan (LAM) and lipid-free arabinomannan (AM) were prepared from *Mycobacterium paratuberculosis*. Purification of LAM was done by ultracentrifugation of the phenol-water-extracted crude polysaccharide, followed by affinity and anion exchange chromatography. AM was purified from the supernatant of the ultracentrifuged polysaccharide or from alkaline-extracted material by gel filtration and anion exchange chromatography. Chemical analysis revealed arabinose and mannose in LAM (1.4:1) and AM (3.5:1) and the presence of palmitic, stearic, and tuberculostearic acids for a total of 7.8% lipid in LAM. Traces of phosphorus were found in the AMs, particularly LAM (0.05%). Nuclear magnetic resonance confirmed the presence of  $\alpha$ -arabinosyl residues and the acylated nature of LAM. LAM exhibited lipid-dependent aggregation, as indicated by a Triton-induced decrease in molecular weight. By using bovine sera, LAM was found to be active in the complement fixation test, whereas AM was inactive and inhibited this activity. Thus, the presence of AM in crude polysaccharide could explain the variable complement fixation results. Triton-dissociated LAM exhibited a precipitin (C1) in common with that of AM, confirming shared determinants. LAM in its lipid-dependent aggregated form, however, exhibited a second precipitin (C2), which may be due to the disparity in antigen size or a novel epitope. The lipid content of LAM rendered it 100 times more effective for coating plates in the enzyme immunoassay than lipid-free AM.

A polysaccharide antigen for the serodiagnosis of paratuberculosis in cattle by the complement fixation test (CFT) was first prepared through phenol-water extraction of *Mycobacterium paratuberculosis* by Annau (3). A similarly extracted polysaccharide antigen was characterized chemically and antigenically by Yugi et al. (46, 47). Subsequent serodiagnosis with this antigen in CFT permitted the detection of a humoral response (37) which correlated well with bacterial load in cattle (17). A direct relationship between humoral response and bacillary burden was also noted in untreated leprosy patients with a polysaccharide antigen (28).

In this laboratory the phenol-water-extracted polysaccharide antigen showed two major precipitins in agar gel immunodiffusion (AGID), suggesting the presence of two antigens. This could complicate any critical evaluation of CFT findings, since test sera can be qualitatively different (i.e., contain different ratios of two antibody populations) than sera used in test calibration. Inconsistent potency between preparations and anticomplementary reactions were also observed.

The purification and chemical characterization of polysaccharide antigens from *M. paratuberculosis* was undertaken to discover the basis for difficulties associated with antigen preparation and to allow the reproducible preparation of antigens for serodiagnosis of paratuberculosis. In addition to the goal of improving the sensitivity and specificity of such test procedures by the use of purified and characterized antigens (38), as has been the case for other antigens from *M. paratuberculosis* (1, 45), the use of defined epitopes should also permit a better understanding of the humoral response as it relates to serodiagnosis and immunopathogenesis of a

mycobacterial disease (14) with some of the spectral manifestations observed in leprosy (30) and tuberculosis (26).

### MATERIALS AND METHODS

**Growth of *M. paratuberculosis*.** Laboratory-adapted strains of *M. paratuberculosis* (strains II, III, IV, V, III+V, C-286, and C-300) from paratuberculosis-infected animals were grown in modified Long synthetic medium for 3 months (3). Bacilli were killed in 3% phenol, washed, and stored frozen at -20°C.

**Phenol-water extraction and preparation of C polysaccharide.** To 100 g of drained bacilli was added 40 ml of water and 60 ml of 90% phenol (3). The slurry was homogenized for 1 to 2 min with a Willems Polytron PT20ST homogenizer (Brinkmann Instruments, Canada Ltd., Toronto), stirred for 30 min, and centrifuged at 30,000  $\times g$  for 30 min at 4°C. Crude (C) polysaccharide was precipitated from the aqueous phase with 4 volumes of ethanol-ether (2:1) chilled in powdered dry ice. After 1 to 2 days in the freezer at -10°C, the precipitate was recovered by low-speed centrifugation, washed with ethanol and ether (-10°C), and dried over Drierite (W. A. Hammond Drierite Co., Xenia, Ohio). Treatment of this crude material with acetic acid and reprecipitation were done as before (3).

**Purification of polysaccharides from phenol-water-extracted C polysaccharide.** The C polysaccharide was ultracentrifuged at 170,000  $\times g$  at 4°C for 3 h in a solution containing 0.1 M Tris (Sigma Chemical Co., St. Louis, Mo.), pH 8.0, 1.0 M NaCl, 0.02% NaN<sub>3</sub> (Tris-NaCl) to obtain S (supernatant) and P (pellet) polysaccharides.

The S polysaccharide was first separated on a column (5.0 by 89.5 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) preequilibrated with Tris-NaCl. Selected pools were subsequently chromatographed on a column (1.6 by 69.5 cm) of DEAE-Sephacel (Pharmacia) preequilibrated with 0.05 M Tris, pH 8.0, and 0.02% NaN<sub>3</sub> (Tris) at 4°C.

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Elution was with Tris and then a 0 to 0.6 M NaCl linear gradient in Tris. The P polysaccharide was chromatographed on a column (5.0 by 25.0 cm) of concanavalin A-Sepharose 4B (ConA-Sepharose; Pharmacia) by stepwise elution with 0.1 M Tris (pH 7.2)–1 mM  $\text{MgCl}_2$ –1 mM  $\text{CaCl}_2$ –1 mM  $\text{MnCl}_2$ –0.02%  $\text{NaN}_3$  (ConA buffer) followed by 0.1 and 0.5 M  $\alpha$ -methylmannoside (Sigma) in ConA buffer, respectively. Selected pools were further chromatographed on DEAE-Sepharose as above. It was necessary, however, to ultracentrifuge the CFT-active pool beforehand in the presence of 0.1 M  $\alpha$ -methylmannoside in Tris buffer to ensure removal of noncovalently associated lipid (see Results). After each step, polysaccharides were dialysed with washed Spectropor tubing (3,500 molecular weight cutoff; Spectrum Medical Industries Inc., Los Angeles, Calif.) and lyophilized.

**Alkaline extraction of AM.** To extract arabinomannan (AM), an acetone-washed mass of bacilli (300 g) was extracted with 0.5 N NaOH (300 ml) at 70 to 75°C under  $\text{N}_2$  for 8 h (28, 29). After neutralization with glacial acetic acid and low-speed centrifugation, polysaccharide was precipitated at 30, 66, and 80% ethanol and recovered by low-speed centrifugation. The 80% precipitate was chromatographed on Sephacryl S-200 and DEAE-Sepharose, with fractions being pooled as for the chromatography of S polysaccharide from phenol-water-extracted material.

**Analytical methods.** Total carbohydrate of unpurified polysaccharides and chromatographic fractions was determined by the phenol-sulfuric acid method (19) with glucose as the standard. The carbohydrate content of purified AMs was determined on weighed lyophilized portions with standards containing appropriate ratios of arabinose to mannose as revealed by the monosaccharide content of the polysaccharides (see Table 1).

The monosaccharide content was determined by high-pressure liquid chromatography (HPLC) of dansyl hydrazones (2, 5) after trifluoroacetic acid (TFA) hydrolysis (16) as well as gas-liquid chromatography (GLC) of alditol acetates (12). A 1.0-mg portion of polysaccharide was treated with 4 N TFA in sealed tubes for 16 h at 100°C. TFA was removed by evaporation, and the sample was dried overnight in vacuo over potassium hydroxide pellets. Reaction with dansyl hydrazine (Sigma) and clean-up on a Seppak C18 cartridge (Waters Associates, Milford, Mass.) were done by the method of Alpenfels (2). Separations were carried out in a Beckman 330 isocratic system with a 210 injector, 153 UV detector at 254 nm, and 110 solvent delivery system (Beckman Instruments Inc., Berkeley, Calif.) using an Altex Ultrasphere-Octadecylsilyl (ODS) (5  $\mu\text{m}$  packing; 4.6 mm inner diameter by 25 cm) analytical column (Beckman), with a Brownlee RP-GU guard column of C-8 (RP-8) (10  $\mu\text{m}$  packing) (Brownlee Labs Inc., Santa Clara, Calif.). The flow rate was 1.0 ml/min. Monosaccharide identity was based on coelution with authentic standards in both the HPLC and GLC procedures.

Qualitative and quantitative analyses of fatty acids were based on (i) thin-layer chromatography (TLC) of hydroxymate derivatives, (ii) HPLC of *p*-nitrobenzyl derivatives, and (iii) spectrophotometric determination of long-chain fatty acids.

The TLC protocol was a modification of that used by Pless et al. (34) and used standards of oleohydroxymate, palmitohydroxymate, and stearohydroxymate synthesized from the fatty acids (Sigma) by the method of Keller and Ballou (24). Succinohydroxymate was also used and was prepared from succinic anhydride (27).

To 10 mg of polysaccharide dried in vacuo over  $\text{P}_2\text{O}_5$  was added 100  $\mu\text{l}$  of alkaline hydroxylamine (27). Standards were 10 mg of glycogen in the absence or presence of 100  $\mu\text{g}$  of the appropriate fatty acid hydroxymate derivatives. To these was also added 100  $\mu\text{l}$  of alkaline hydroxymate. After 20 min, 250  $\mu\text{l}$  of ethanol-ether (3:1) was added, and after a further 20 min, 1 ml of the ethanol-ether was added as well as 0.1 g of Amberlite IR-120 (Mallinckrodt Chemical Works, St. Louis, Mo.) which had been successively washed with methanol, water, 0.1 N HCl, and water. After low-speed centrifugation, the supernatant was collected and the residue was rinsed with 1 ml of ethanol-ether. The pooled extracts were evaporated under  $\text{N}_2$ , and the residue was taken up in 200  $\mu\text{l}$  of ethanol-ether (3:1), centrifuged, transferred, evaporated, and redissolved in 40  $\mu\text{l}$  of ethanol-ether. This was again centrifuged, and the whole amount as well as hydroxymate standards were spotted on a glass-backed 0.25-mm silica plate (EM Industries, Inc., Gibbstown, N.J.). The plate was developed with benzene-methanol-formic acid (60:40:1) and, after drying, sprayed with 5%  $\text{FeCl}_3$  in ethanol.

Long-chain fatty acids were determined by reverse-phase HPLC of *p*-nitrobenzyl derivatives (25) after alkaline hydrolysis of fatty acids. A 10-mg amount of polysaccharide was treated with 8.0 ml of 0.5 N NaOH under  $\text{N}_2$  for 1 h at 70°C. Following acidification with 1.2 ml of 4.0 N HCl to give a pH of 2, 37.8 ml of chloroform-methanol (2:1) was added, and fatty acids were partitioned into the lower phase after thorough shaking (21). After the chloroform was removed by evaporation, the residue was taken up in methylene chloride, and a portion was reacted with a molar excess of *O*-*p*-nitrobenzyl-*N,N'*-diisopropylisourea (Regis Chemical Co., Morton Grove, Ill.) in methylene chloride at 80°C for 2 h (25). A portion was injected into the Beckman HPLC fitted with the Altex Ultrasphere-ODS column described above. The flow rate was 1.0 ml/min, and detection was at 254 nm. The kind donation of an authentic standard of tuberculostearic acid as the methyl ester from C. Asselineau, Centre de Recherche de Biochimie et de Génétique Cellulaires du C.N.R.S., Toulouse, France, is gratefully acknowledged. Quantitative analysis was done by comparing peak areas with those of alkaline-treated and -extracted fatty acid standards in the presence of 10 mg of glycogen.

A quantitative spectrophotometric determination of long-chain fatty acids in the polysaccharides was also done by the method of Duncombe (20) with palmitic or stearic acid as the standard.

The presence of succinic acid was tested by reacting alkaline-treated polysaccharide extracts with *p*-nitrobenzyl bromide after neutralization in the presence of crown ether, followed by HPLC separation on a silica absorption column (23). Polysaccharide (10 mg) was treated with 0.2 N NaOH for 1 h at 70°C under  $\text{N}_2$ . After neutralization with 0.4 g of Amberlite IR-120, methanol was added to make a total of 3 ml of extract which contained 50% methanol. After low-speed centrifugation, the Amberlite was washed successively with 2 ml of 30% methanol and 2 ml of water, interspersed with centrifugations. To a portion of the pooled extracts was added 3 drops of 1% phenolphthalein in methanol. This was neutralized with 0.1 N KOH in methanol in a Teflon-lined reaction vial and evaporated with  $\text{N}_2$ , followed by the addition of 3 ml of acetonitrile. Then 50  $\mu\text{l}$  of 0.01 M 18-crown-6-ether (Regis Chemical Co.) in acetonitrile and 50  $\mu\text{l}$  (5  $\mu\text{mol}$ ) of *p*-nitrobenzyl bromide (Aldrich Chemical Co., Milwaukee, Wis.) in acetonitrile were added. After sonication for 2 min, the reaction mixture was heated for 15 min at

80°C, decanted, evaporated with N<sub>2</sub>, and taken up in the mobile phase (heptane-chloroform-methanol, 83.5:12.5:4). Separation was on a Spherosorb column (HPLC Technology Ltd., Toronto; 5 µm, 4.6 cm inner diameter by 25 cm) in the Beckman HPLC at 1.0 ml/min, and detection was at 254 nm.

The Bio-Rad assay for protein (11) was standardized with bovine serum albumin (Sigma). Nitrogen was determined in the 5 to 30 µg range by the Nessler reaction (44). Total phosphorus was determined with reagents of the EM diagnostics kit for inorganic phosphate (22, 36) (EM Labs, Inc., Elmsford, N.Y.) after a modified wet digestion technique (4). Polysaccharide (10 mg) in 1 ml of water along with KH<sub>2</sub>PO<sub>4</sub> and glucose 6-phosphate as standards were digested for 30 min with 1 ml of concentrated HNO<sub>3</sub> in 15-ml tubes on a Gallenkamp apparatus. Digestion with 0.5 ml of 70 to 72% perchloric acid was performed under heat, and portions were analyzed.

**NMR.** Proton nuclear magnetic resonance (NMR) spectra were recorded in D<sub>2</sub>O solution at 500 MHz for a spectral width of 5 KHz digitized over 16,000 data points. The probe temperature was 310 K, and chemical shifts were referenced to internal acetone ( $\delta = 2.225$  ppm). Carbon-13 spectra were recorded at 125.7 MHz on a Bruker AM-500 spectrometer with quadrature detection, using a  $\pi/2$  pulse (8.5 µs), a 25-KHz spectral width, and a 32,000-point data set. The probe temperature was 310 K, and chemical shifts were referenced to internal 1,4-dioxane (1% vol/vol) ( $\delta = 67.4$  ppm). To minimize heating effects, gated broadband decoupling of 1 W was used to establish nuclear Overhauser experiment build-up during a delay period of 0.7 s, followed by approximately 1-W composite pulse decoupling (WALTZ) (39) during acquisition (0.65 s). Proton-decoupled distortionless enhancement by polarization transfer (DEPT) experiments (18) were performed for a 12.5-KHz spectral width, using a  $3\pi/2$  proton pulse and pulse delay of 3.4 ms to distinguish CH and CH<sub>2</sub> resonances.

**Enzymatic digestion and trituration.** To determine the effect of possible protein, nucleic acid, or lipid contamination on antigenic activity, samples of lipoarabinomannan (LAM) were treated with the appropriate enzyme or triturated with organic solvent. To two lyophilized 1.0-mg portions were added RNase (from bovine pancreas) and DNase I (from bovine pancreas) at 0.05 mg of each per ml of 0.01 M sodium phosphate, pH 7.0 (7). To a second 1.0-mg portion was added protease (proteinase K from *Tritirachium album*, type XI) at a concentration of 0.1 mg/ml of sodium phosphate, pH 7.0. The enzymes were purchased from Sigma. The nuclease digestions were for 2 h at room temperature, whereas the protease digestion and a control (LAM in buffer) were at 37°C for 2 h. All four samples were diluted to approximately 7 ml with water and ultracentrifuged at  $170,000 \times g$  for 3 h. The pellet was taken up in water and lyophilized. To a 10-mg lyophilized portion of LAM was added 2 ml of chloroform-methanol (2:1). This was vortexed and centrifuged. This procedure was repeated, and the sedimented LAM was dried with N<sub>2</sub>.

**Serological methods.** AGID plates were set up with 1% Noble agar (Difco Laboratories, Detroit, Mich.) in 0.1 M Tris (pH 7.3)–1.0 M NaCl–0.02% NaN<sub>3</sub> with and without incorporated 0.1% Triton X-100 (Sigma). Undiluted serum (25 µl) from a paratuberculosis-infected cow with a high CFT titer was placed in the center well, and surrounding wells contained various quantities of antigen. Precipitin lines were read after 48 h of incubation at room temperature. CFT tests with bovine sera were conducted as described previously (17) at 0.1 mg of polysaccharide per ml. The enzyme

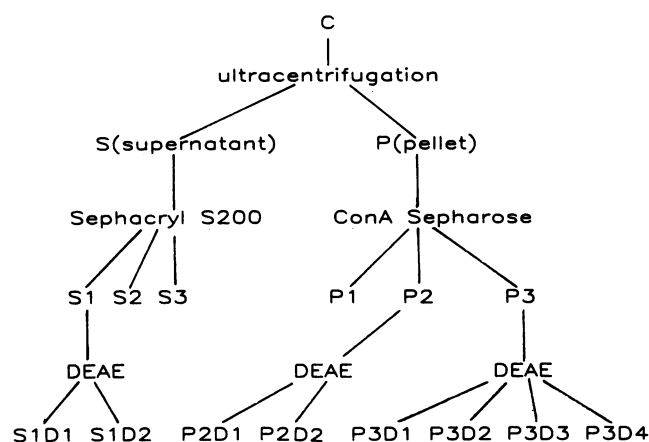


FIG. 1. Purification protocol for polysaccharides. Phenol-water-extracted C polysaccharide was ultracentrifuged to give S and P polysaccharides. S polysaccharide was separated into S1, S2, and S3 on Sephacryl S-200, and S1 was subsequently chromatographed on DEAE-Sephacel to give S1D1 and S1D2. The P polysaccharide was chromatographed on ConA-Sepharose to give P1, P2, and P3, and the latter two pools were subsequently chromatographed on DEAE-Sephacel to yield the six pools indicated in the figure. The S1D1 pool proved to contain lipid-free AM and P3D2 consisted of LAM.

immunoassay followed a protocol developed by Nielsen and Wright (31). Antigen was coated overnight onto polystyrene Titertek microplates (Flow Laboratories, McLean, Va.) at various concentrations in 0.06 M carbonate buffer, pH 9.6. After the plates were washed with 0.01 M sodium phosphate, pH 7.2, containing 0.05% Tween 20 (PBS-T), diluted serum (1/200) was added and incubated for 3 h at room temperature. The plates were washed with PBS-T, and appropriately diluted and absorbed rabbit anti-bovine immunoglobulin G (IgG) (heavy and light chains) conjugated with horseradish peroxidase (Cappel Laboratories, Westchester, Pa.) was added to the plates. After a further 1 h of room temperature incubation, substrate, consisting of H<sub>2</sub>O<sub>2</sub> with 2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma) as hydrogen donor, was added (add 0.1 ml of 3% H<sub>2</sub>O<sub>2</sub> and 0.5 ml of 40 mM ABTS to 20 ml of 0.05 M citrate buffer, pH 5.0). Plates were read at 414 nm after 8 min with a Titertek Multiskan Microplate reader (Flow Laboratories) interfaced to a computer (31).

**HPLC gel filtration of polysaccharide.** A 1.0-mg portion of polysaccharide in 1.0 ml of 0.05 M Tris (pH 8.0)–0.1 M NaCl–0.02% NaN<sub>3</sub> was injected at 1.0 ml/min into the Beckman HPLC fitted with a TSK-gel G4000 SW column (7.5 mm by 30.0 cm) preequilibrated with the same buffer in the absence or presence of 0.1% Triton X-100.

## RESULTS

**Preparation and purification of polysaccharides.** Strain V was selected from seven laboratory strains on the basis of bacterial mass yield and ease of reconstitution of the polysaccharide in aqueous buffers. An approximate yield of 230 mg of C polysaccharide from 100 g (wet weight) of strain V bacilli was obtained.

The purification protocol is summarized in Fig. 1. At each stage of the procedure, the progressively purified polysaccharides were monitored for CFT activity. Ultracentrifugation of the C polysaccharide yielded S and P polysac-

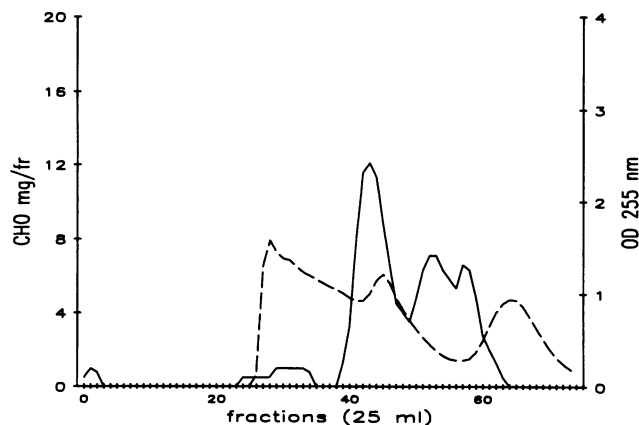


FIG. 2. Sephacryl S-200 gel filtration of ultracentrifugation supernatant from phenol-water-extracted polysaccharide. To a column (5.0 by 89.5 cm) was applied 169.1 mg of supernatant polysaccharide. Elution was with Tris-NaCl. Dextran T70, T40, T20, and T10 peaks occurred at fractions (fr) 29, 34, 40, and 45, respectively. Collected pools were 40–49 (S1), 50–57 (S2), and 58–65 (S3). Symbols: —, carbohydrate (CHO); - - -, OD<sub>255</sub>

charides, which were CFT inactive and CFT active, respectively. Gel filtration on Sephacryl S-200 may effectively substitute for ultracentrifugation; nonexcluded and excluded peaks would correspond to S and P polysaccharides, respectively.

S polysaccharide was fractionated on Sephacryl S-200 into S1, S2, and S3 (Fig. 2). The S1 polysaccharide, occurring in the 40–49 pool, was the largest and exhibited a molecular weight of approximately 14,000 on the basis of dextran standards. This polysaccharide was further processed, whereas the S2 and S3 polysaccharide pools have yet to be characterized. Alkaline-extracted material was chromatographed, and the S1 polysaccharide pool was similarly selected. On DEAE-Sephacel, S1 polysaccharide from phenol-water- or alkaline-extracted material was resolved into unretained polysaccharide (S1D1) and salt-eluted UV-absorbing material (S1D2) (data not shown). S1D1 from

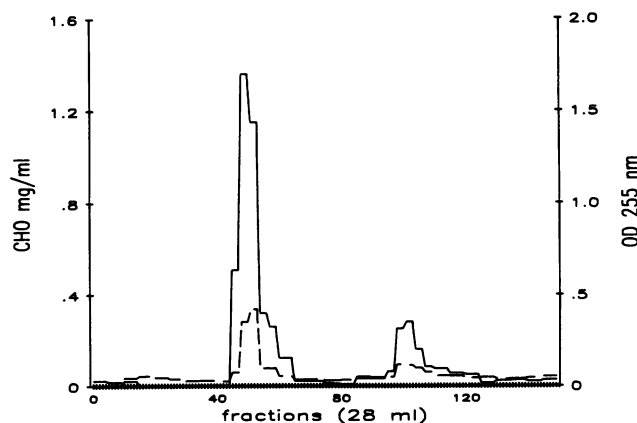


FIG. 3. ConA-Sepharose affinity chromatography of P (pellet) from phenol-water-extracted polysaccharide. To a column (5 by 25 cm) was applied 676 mg of the CFT-active ultracentrifugation pellet. Elution was stepwise with ConA buffer at the beginning and with ConA buffer plus 0.1 and 0.5 M  $\alpha$ -methylmannoside at fraction 37 and 84 to give collected pools 1–36 (P1), 37–64 (P2), and 84–124 (P3). Symbols: see Fig. 2 legend.

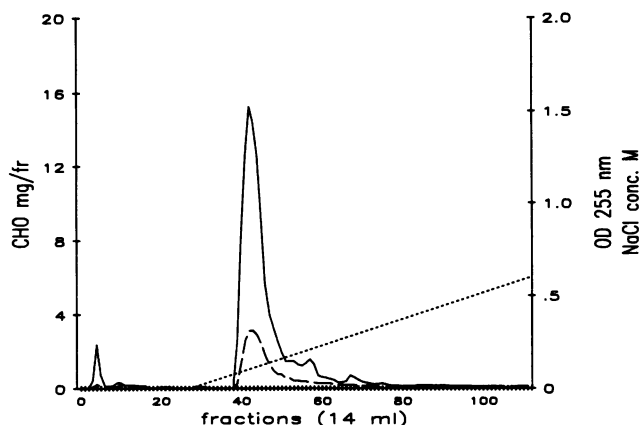


FIG. 4. DEAE-Sephacel chromatography of CFT-active polysaccharide from ConA-Sepharose pool P3 (Fig. 3) after ultracentrifugation in 0.1 M  $\alpha$ -methyl mannoside. To a column (1.6 by 69.5 cm) was applied 150.6 mg of polysaccharide. Elution was done as described in the legend to Fig. 3. Collected pools are 1–8 (P3D1), 40–53 (P3D2), 54–63 (P3D3), and 64–75 (P3D4). Symbols: —, carbohydrate (CHO); - - -, OD<sub>255</sub>; - · - ·, NaCl concentration.

phenol-water-extracted (S1D1-PW) or alkaline-extracted (S1D1-A) polysaccharide was CFT inactive.

P polysaccharide was resolved on ConA-Sepharose into P1, P2, and P3 pools (Fig. 3), of which P1 and P2 were CFT inactive and P3 was strongly CFT active. The P2 pool was further separated on DEAE-Sephacel into unretained P2D1 and some salt-eluted polysaccharide (approximately 1/3 of mass) spread out over the NaCl gradient (P2D2) (data not shown). The P3 polysaccharide was insoluble in Tris buffer unless  $\alpha$ -methylmannoside was included. After ultracentrifugation of P3 thus solubilized, the pellet was Tris soluble, suggesting that noncovalently associated lipid had been removed in the supernatant. Indeed chloroform-methanol extraction had the same effect. The presence of this lipid in former crude preparations could be one of the factors contributing to anticomplementary effects in the CFT. Chromatography of P3 on DEAE-Sephacel yielded the unretained polysaccharide pool (P3D1) and three salt-eluted polysaccharide pools (P3D2, P3D3, and P3D4) (Fig. 4). The CFT titers of these latter three pools were found to be 1/160, 1/320, and 1/320, respectively, compared with 1/20 for the original P polysaccharide. This both reflects a substantial increase in specific activity after purification and suggests a relationship between molecular charge and antigenic activity.

**Chemical analysis.** Table 1 depicts the monosaccharide

TABLE 1. Monosaccharide composition of *M. paratuberculosis* antigens<sup>a</sup>

Polysaccharide	Mean mol% $\pm$ SD (no. of trials)			Arabinose/ mannose
	Arabinose	Mannose	Glucose	
P	0.10 (1)	0.13 (1)	0.77 (1)	0.8
P2	ND	ND	1.00 (1)	
S1D1-PW	0.78 $\pm$ 0.05 (3)	0.21 $\pm$ 0.04 (3)	Tr (1)	3.7
S1D1-A	0.76 $\pm$ 0.13 (2)	0.24 $\pm$ 0.13 (2)		3.2
P3D2	0.58 $\pm$ 0.12 (6)	0.41 $\pm$ 0.11 (6)	Tr (1)	1.4

<sup>a</sup> Composition as mean mole fraction of monosaccharides for selected antigen pools. The total number of determinations by HPLC detection of dansyl hydrazone or GLC detection of alditol acetates is shown in parentheses. Tr, Trace (mole fraction less than 0.01); ND, not detected.

content of the polysaccharides. These showed that the initial CFT-active P polysaccharide was a mixture of glucan and AM in an approximately 3:1 ratio. These cannot be separated by ultracentrifugation since glucan is also pelleted (13). Subsequent chromatography on ConA-Sepharose and DEAE-Sepharose purified a glucan (P2) and an AM (P3D2). Chromatographically purified phenol-water supernatant and alkaline-extracted polysaccharides (S1D1-PW and S1D1-A) were also AMs which showed comparable but different arabinose-mannose ratios than P3D2.

The various AMs were tested for the presence of esterified acyl groups by TLC of hydroxymates (Fig. 5). The CFT-active AM (P3D2) exhibited hydrophobic hydroxymates which migrated similarly to long-chain fatty acid hydroxymates, whereas CFT-inactive AMs (S1D1-PW and S1D1-A) were devoid of these hydrophobic hydroxymates. The spot corresponding to hydrophobic hydroxymates from P3D2 was likely retarded by overloading, which was done on purpose to scout for possible succinohydroxymate, the presence of which was suggested by lower spots in this TLC experiment. (The top and bottom weaker spots due to succinohydroxymate are believed to be elution artifacts caused by glycogen or a hydroxylamine excess.) However, the HPLC experiments on *p*-nitrobenzyl derivatives of succinic acid were unable to detect any esterified succinic acid. On the other hand, HPLC of the *p*-nitrobenzyl derivative of fatty acids of CFT-active AM (P3D2) revealed peaks corresponding to authentic standards of palmitic, stearic, and tuberculostearic acids (Fig. 6), as well as two faster-eluting uncharacterized substances. At this point the CFT-active (P3D2) and -inactive (S1D1-PW and S1D1-A) AMs were designated LAM and lipid-free AM, respectively. The composition of the AMs determined on lyophilized material is given in Table 2. All were mainly carbohydrate with small or trace amounts of protein, nitrogen, and phosphorus. The most noteworthy difference between LAM and AM was the presence of 7.8% lipid in LAM exclusively, composed of 4.3% palmitic, 0.3% stearic, and 3.2% tuberculostearic acids, as determined by coelution of authentic standards. The presence of protein and the higher amount of phosphorus in LAM are two further differences between it and AM.

The UV-absorbing material (S1D2) was clearly separated from the AM polysaccharides (S1D1-PW and S1D1-A) (data



FIG. 5. TLC of fatty acid hydroxymates from polysaccharides. A through F, 100- $\mu$ g standards plus 10 mg of glycogen: A, glycogen alone; B, succinohydroxymate; C, stearohydroxymate; D, palmitohydroxymate; E, oleohydroxymate; F, all three fatty acid and succinic acid hydroxymates. G through I, 10-mg polysaccharide samples: G, S1D1-A; H, P3D2; I, S1D1-PW. J, All three fatty acid and succinic acid hydroxymates in the absence of glycogen.

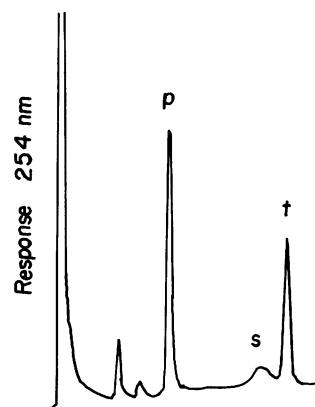


FIG. 6. HPLC separation of nitrobenzyl-derivatized fatty acid extract from hydrolyzed P3D2 LAM, revealing peaks corresponding to palmitic acid (p), stearic acid (s), and tuberculostearic acid (t) with retention times of 23.0 min (p), 40.35 min (s), and 45.45 min (t).

not shown). However, since UV absorption was coincident with the polysaccharide mass in Fig. 3 and 4, it is unknown how much this was due to distinct substances or to UV absorption by the polysaccharide. On scanning from 400 to 200 nm, LAM exhibited an absorption peak at 210 nm with a slight shoulder between 280 and 250 nm. Chemical analysis was not done on the other ConA-Sepharose pools, although these are likely AM (P3D1) or LAM (P3D3 or P3D4) from the AGID, CFT, and chromatographic evidence. The P2D1 and P2D2 glucan pools were not further investigated.

**NMR spectra.** The 500-MHz proton spectra of AM and deacylated LAM were identical, indicating that LAM is essentially an acylated form of AM (Fig. 7B and C). In agreement with this, the proton spectra of LAM indicates the presence of aliphatic protons at  $\sim 1.0$  ppm (Fig. 7A), as was found for the mannan from *Micrococcus lysodeikticus* (34). The  $^{13}\text{C}$  spectrum of the LAM is shown in Fig. 8. The spectrum records only C atoms carrying protons, and as displayed, methine and methyl carbons are phased positive, while methylene carbon atoms are phased negative. Thus, the primary carbons of arabinose or mannose residues appear as negatively phased signals. It is immediately obvious that there were two sets of primary hydroxyl groups present in the polymer, those at  $\sim 62.0$  ppm representing unsubstituted  $-\text{CH}_2\text{OH}$  groups and those between 67.0 and 70.0 ppm representing primary hydroxy groups that are glycosylated. The anomeric carbon atoms show major reso-

TABLE 2. Chemical composition of AMs from *M. paratuberculosis*

Component	Composition (mean % by wt $\pm$ SD) <sup>a</sup>		
	LAM P3D2-PW	AM S1D1-PW	AM S1D1-A
Carbohydrate	73.9 $\pm$ 9.6 (3)	88.1 (1)	86.8 $\pm$ 1.5 (2)
Lipid	7.8 $\pm$ 1.4 (5) <sup>b</sup>	— (2) <sup>c</sup>	— (2) <sup>c</sup>
Protein	1.35 (2)	— (1) <sup>d</sup>	0.07 (1)
Nitrogen	0.87 (1)	0.33 (1)	0.11 (1)
Phosphorus	0.05 (1)	0.02 (1)	0.01 (1)

<sup>a</sup> Percentage of lyophilized weight, moisture unaccounted for; the number of analyses is shown in parentheses.

<sup>b</sup> As determined by HPLC and spectrophotometry.

<sup>c</sup> No TLC evidence of hydrophobic hydroxymates.

<sup>d</sup> No protein detected.

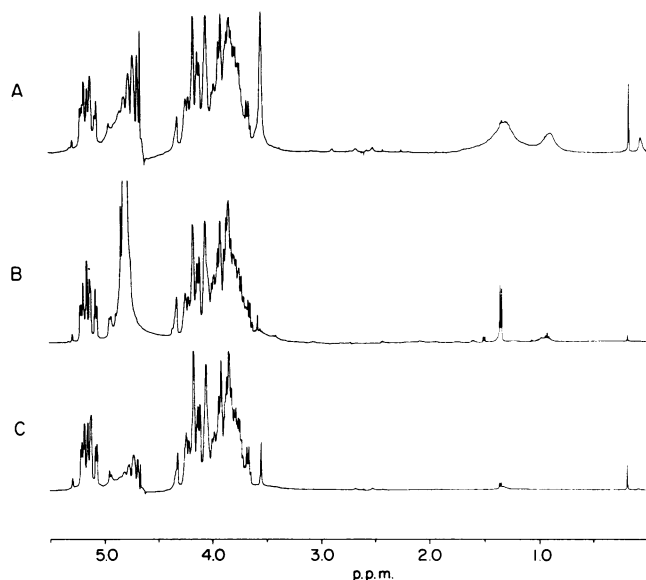


FIG. 7. Proton NMR spectra of AMs at 500 MHz. (A) Phenol-water-extracted LAM; (B) phenol-water-extracted AM; (C) alkaline-deacylated LAM.

nances of  $\sim 108.0$ ,  $103.0$ , and  $98.5$  ppm, with minor intensity resonances at  $107.5$ ,  $106.0$ , and  $102.0$  ppm. The lowest field anomeric signals corresponded to  $\alpha$ -linked arabinofuranosyl residues (10), while all of the signals below  $105$  ppm may originate from mannopyranosyl,  $\alpha$ -arabinopyranosyl, or  $\beta$ -arabinopyranosyl residues (8–10). From a cursory inspection of the  $^{13}\text{C}$  spectrum, it can be concluded that the polysaccharide contains a large proportion of  $\alpha$ -arabinofuranosyl residues which are most likely glycosylated at the primary position. These conclusions are largely in agreement with those reached by Weber and Gray (43).

**Antigenic activity.** The P polysaccharide (as well as the original C polysaccharide) was CFT active and exhibited C1

and C2 precipitins (Fig. 9). The C1 occurred close to and curved slightly around the antiserum well, whereas C2 did the same relative to the antigen well (Fig. 9A). The P polysaccharide was ultimately resolved into CFT-inactive AM showing C1 precipitin and CFT-active LAM showing C2 precipitin. It should be noted that the presence of C1 persisted in LAM until DEAE-Sephacel chromatography was performed, indicating that this step was necessary to remove small amounts of AM. A third weak precipitin was observed in the P2 polysaccharide after ConA-Sepharose chromatography, which was thought to be due to glucan. The smaller amounts (at least 10-fold) of LAM were required to show C2 precipitin than P polysaccharide (Fig. 9). It was also noted that the C1 precipitin from both phenol-water- and alkaline-extracted AM as well as deacylated LAM showed fused lines of identity which appeared to show a partial identity spur with C2 precipitin (Fig. 9B and C). With the incorporation of 0.1% Triton X-100 into the agar, the C2 precipitin of LAM vanished and a precipitin appeared which fused with the C1 precipitin of AM (Fig. 9E and F). Preliminary evidence from HPLC gel filtration showed a Triton-induced decrease in molecular weight from 1,025,000 to 71,000. Removal of Triton by dialysis and rechromatography on the HPLC column in the absence of Triton to collect the excluded peak resulted in a LAM that was again capable of exhibiting the C2 precipitin (Fig. 9C). In the case of P polysaccharide which shows both C1 and C2, when Triton was incorporated, C2 vanished and C1 alone was evident (Fig. 9D).

Further experimentation demonstrated that CFT-inactive AM was inhibitory to the CFT activity of LAM. A bovine serum, demonstrating an optimum titer of  $1/40$  with  $2\text{ }\mu\text{g}$  of LAM per ml, was seen to show a decline to  $1/10$  in the presence of  $2\text{ }\mu\text{g}$  of AM per ml, and at  $10\text{ }\mu\text{g}$  of AM per ml activity was abolished. Similar inhibitory activity was observed with added LAM, but this was believed to represent a prozone effect.

Finally, LAM and AM were tested for their activity in an enzyme immunoassay. LAM showed optimal activity at a coating concentration of  $1\text{ }\mu\text{g/ml}$  on polystyrene plates,

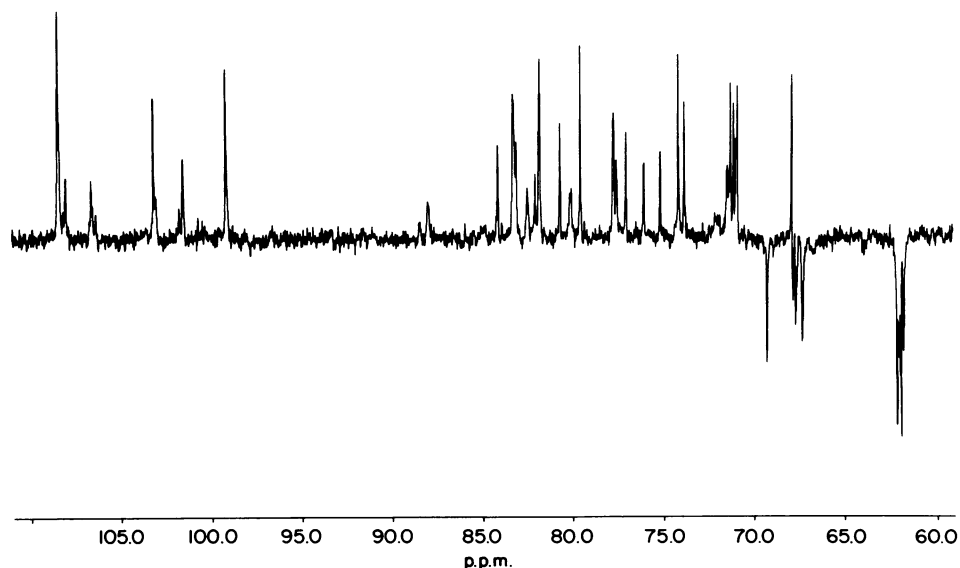


FIG. 8. Carbon-13 NMR of LAM.

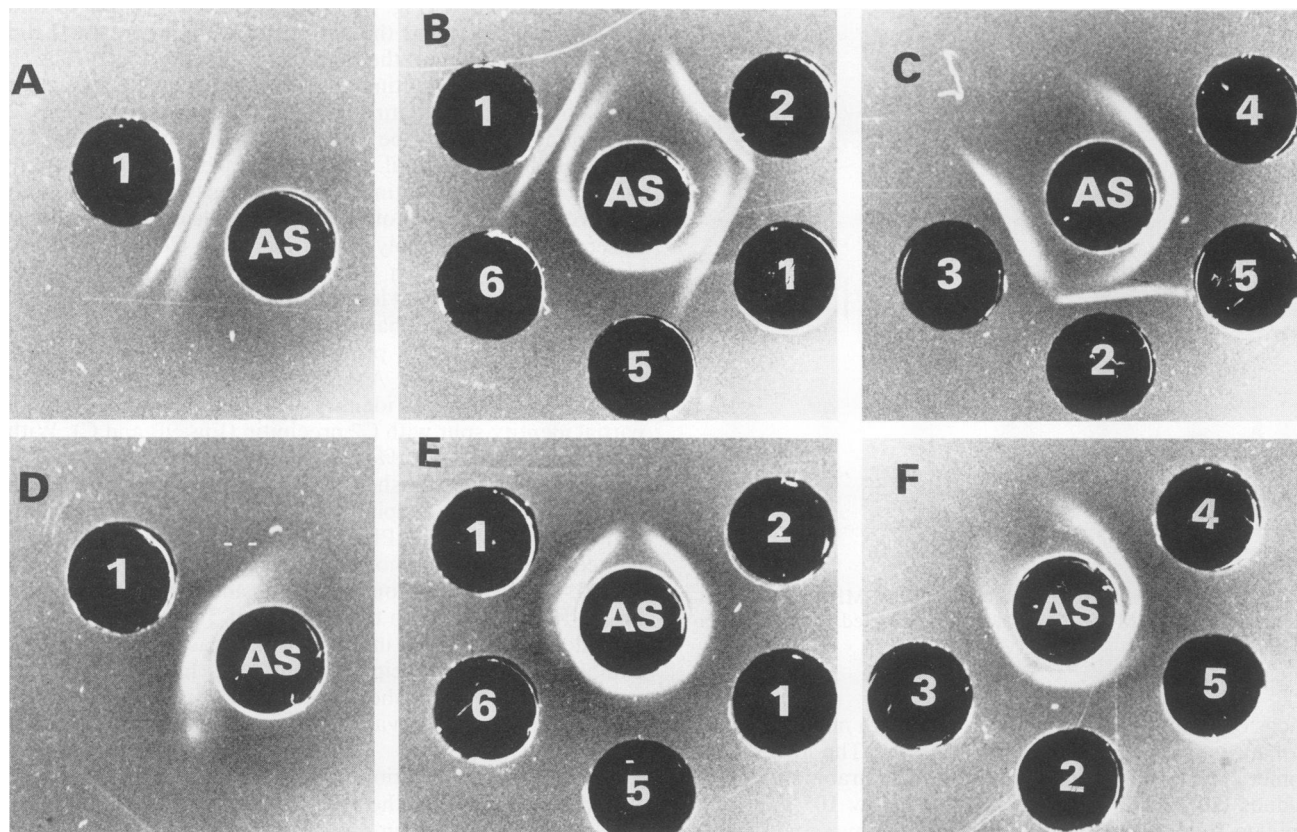


FIG. 9. AGID of polysaccharide antigens. Well 1, 70  $\mu$ g of P polysaccharide; well 2, 7  $\mu$ g of LAM; well 3, 7  $\mu$ g of LAM after Triton X-100 dissociation and subsequent removal of Triton X-100; well 4, 1  $\mu$ g of deacylated LAM; well 5, phenol-water-extracted AM; well 6, alkaline-extracted AM; center wells (AS) contain 25  $\mu$ l of undiluted serum from an infected cow. (A, B, and C) No Triton; (D, E, and F) 0.1% Triton X-100 incorporated into the agar.

whereas AM showed apparent poor coating even at 100  $\mu$ g/ml. With LAM, a CFT-negative serum from an uninfected animal showed an OD of 0.092, and a CFT-positive serum from a paratuberculosis-infected animal showed an OD of 1.750.

## DISCUSSION

This study has demonstrated the existence of two AMs in *M. paratuberculosis*: LAM containing esterified fatty acids and lipid-free AM. The latter was obtained from the ultracentrifugal supernatant of phenol-water-extracted bacilli and the alkaline extract of bacilli, or alternatively from alkaline deacylated LAM. It resembles alkaline extracts of *M. tuberculosis* (6, 29) and *M. smegmatis* (28) and the culture filtrate AM from *M. tuberculosis* (15). In this report, however, it is evident that phenol-water extraction of bacilli results in the conservation of LAM, which resembles similarly extracted material from *M. tuberculosis* and *M. smegmatis* (32, 33, 40–42).

In addition to the presence of arabinose and mannose in LAM and AM and long-chain fatty acids in LAM, it should be noted that the lower spots in Fig. 6 and the unidentified peaks in Fig. 7 could conceivably represent low amounts of other lower-molecular-weight fatty acids. The covalent esterification of the fatty acids is supported by (i) the failure to remove these fatty acids by chloroform-methanol (2:1)

trituration of untreated LAM, whereas they were extracted from acidified alkaline hydrolysates of LAM, and (ii) the presence of hydroxamate esters after treatment of LAM with alkaline hydroxylamine. NMR studies support the chemical analysis, revealing LAM as an acylated form of AM and confirming the presence of  $\alpha$ -arabinofuranosyl and probably mannopyranosyl residues.

Several studies have claimed esterified succinate as a cause for the anionic nature of various polysaccharides from mycobacteria and *Micrococcus* spp. (24, 34, 35, 43). Although there was some suggestion for the presence of succinate in LAM from a TLC experiment, it was not detected by HPLC. Other possibilities for explaining the anionic nature of LAM include the presence of phosphate, protein, or other nitrogenous substances. Trace amounts of phosphate were found in alkaline-extracted and phenol-water-extracted AM and particularly in LAM (0.05%), but no effort has been made to determine whether phosphate is an integral part of these structures.

It was evident from this work that the presence of AM in the C polysaccharide led to the variable potency and multiple precipitin activity which were part of the original impetus for this study. AM was responsible for the C1 precipitin and CFT-inhibitory activity, which, if occurring in different proportions between preparations, would inhibit the CFT activity of LAM to various degrees. While the bulk of AM was removed through ultracentrifugation, the persistence of C1 precipitin until DEAE-Sephacel chromatography seemed

to be due to the removal of the last vestige of AM through this step.

The fact that the AM C1 precipitin fused as a line of identity to that for Triton-dissociated LAM means that the C1 precipitin measured an anti-AM antibody, in light of the evidence that alkaline-extracted AM was essentially free of other constituents (29). The strong alkaline conditions would, in fact, be expected to hydrolyze all ester bonds. The sharing of determinants between AM and LAM is also supported by the inhibitory effect of AM in CFT, as also noted by Yugi et al. (47).

The existence of the distinct C2 precipitin for LAM in the absence of Triton is intriguing. Although AGID evidence suggests that LAM at least shares a determinant with AM, it seems possible that C2 could arise due to a novel epitope which arises through lipid-dependent aggregation. Alternatively, C2 precipitin could be due to the same AM determinant as C1, arising from an anomalous formation of a separate precipitin due to a wide disparity in molecular size. At present, both alternatives seem possible.

One of the most significant and encouraging findings was the observation that LAM was active in the enzyme immunoassay at a much lower concentration than was possible with AM, permitting conservation of antigen. Alternatively, a fatty acid could be attached to AM as has been done previously (42), and this synthetic LAM could be used in enzyme immunoassays.

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